

The University of Chicago

THE PROTEINS OF THE BRAIN

A STUDY OF THEIR PROPERTIES
AND AN ATTEMPT TO PREPARE
THEM, LIPOID-FREE

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE PHYSICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

1936

By

EDNA RUTH MAIN

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PART I
HISTORICAL INTRODUCTION

In the investigations of the chemical composition of the brain which have extended from the latter part of the eighteenth century up to the present time, studies of the lipoidal constituents have predominated. Prior to 1793, it was believed that the brain consisted of oily and wax-like substances. Fourcroy¹ reported in 1793 that a protein-like substance was one of the essential constituents of the brain. He obtained it from an aqueous emulsion of brain tissue by coagulation by heat or treatment with acids. In 1811 Vauquelin² carried out more extensive studies in which he extracted ground brain with boiling alcohol. The insoluble material remaining contained the membranous material or neurolemma and a coagulated protein which he believed was identical with albumin. This substance swelled with water and readily dissolved in potassium hydroxide solution with no evolution of ammonia, a fact which distinguished it from casein. A white, flocculent precipitate was obtained when the solution was treated with acids. The presence of sulfur was indicated by the dark brown precipitate formed upon addition of lead acetate to the solution. Vauquelin also showed that a protein, soluble in alkali, was present, although in different proportions, in the medulla, spinal cord and the nerves. He believed that some kind of union existed between the fats and the protein and suggested that the function of the protein was to act as a cement for the other constituents. Frémy,³ in 1841, stated that the cerebral material was composed of 7 parts of albumin, 5 parts of fat and 80 parts of water. He showed that the same fats and proteins occurred in both the white and gray matter, but in regard to the properties of the protein, merely stated that it was insoluble in water, alcohol and ether. Petrovsky⁴ analyzed the white and gray matter

¹A. F. Fourcroy, Ann. chim., 16, 282 (1793).

²Vauquelin, Ann. chim., 81, 37 (1812).

³E. Frémy, Ann. chim. phys., 2 (3rd series), 463 (1941).

⁴D. Petrovsky, Arch. ges. Physiol. (Pflügers), 7, 367 (1873).

of the brain separately and found in both a protein which was soluble in dilute solutions of sodium chloride but which was precipitated by a saturated salt solution. The gray matter contained in addition, an albumin which coagulated at 75°. Baumstark⁵ found, in the residue remaining after extraction of the brain with lipid solvents, nuclein, neurokeratin, albumin and connective tissue. The albumin and connective tissue could be removed from the mixture by peptic digestion. The nuclein could be dissolved in alkali leaving the neurokeratin. He also found a small amount of soluble albumin in the water extract which contained the organic extractives.

The proteins described in the early literature were apparently mixtures of protein and lipid in varying proportions which depended upon the method of preparation. Thudichum⁶ was the first to emphasize the necessity of exhaustive extraction with lipid solvents in order to make an effective separation. He extracted two proteins from brain tissue with dilute solutions of salts. One of them gave the usual reactions of serum albumin. The other had the properties of a globulin and resembled the stroma of blood cells. Because of its function as the cell framework, he classified it a plastin and to distinguish it from the plastins of other organs, he called it neuroplastin. The neuroplastin made up by far the greatest part of the insoluble material remaining after thorough extraction of dehydrated brain tissue with ether and alcohol. Less than one-eighth of this residue consisted of albumin, fibrin, cytophosphatides (nucleins) from the cell nuclei, sheaths of the nerve fibers and the tissues which composed the capillaries. Thudichum investigated the hydrolytic products of the neuroplastin. The protein was heated with barium hydroxide at 180° for 6 hours. He was able to identify as products of hydrolysis, acetic, oxalic and phosphoric acids, ammonia, tyrosine, leucine, an isomer of leucine which he termed glyco-leucine, and alkaloids. The latter were not identified individually, but included various nitrogenous bases which were precipitable by phosphotungstic acid.

Thudichum's chief interest was in the lipoids. At about

⁵F. Baumstark, *Z. Physiol. Chem.*, 9, 145 (1885).

⁶J. Ludwig W. Thudichum, "Die Chemische Konstitution des Gehirns des Menschen und der Tiere," Tübingen, 1901.

the same period Halliburton⁷ became interested in the protein constituents and published the first account of studies dealing only with the proteins of the nervous tissue. He came to the conclusion that three different proteins were present. He separated the proteins from extracts made with dilute salt solutions by the principle of fractional heat coagulation. The fraction, which flocculated when the extracts were heated to 47°, resembled cell globulin. It was precipitated from solution by magnesium sulfate in a concentration of 30% and in the absence of salts was not precipitated by acetic acid. It contained no phosphorus. A fraction which he called a neuroalbumin coagulated at 56-60°. It required a high concentration of magnesium sulfate for its complete precipitation. It could also be obtained by precipitation of an aqueous extract of brain tissue with acetic acid. It contained 0.5% phosphorus and after subjection to peptic digestion there remained an insoluble residue of nuclein. The third fraction flocculated at 71-75°. This fraction required saturation with magnesium sulfate for complete precipitation and was not precipitable by dilute acids. It contained no phosphorus. A very small quantity of the fraction which coagulated at 56-60° was found in the white matter.

In his studies of the soluble proteins of the brain, McGregor (see below) could not find temperatures at which excessive coagulation occurred. Since coagulation appeared to him to be a progressive process, he concluded that Halliburton was dealing with one protein rather than with three. Since it is now known that the temperature of coagulation depends upon the concentration of the protein, salts, hydrogen ion and upon the rate of heating, it is evident that without controlling these factors, coagulation temperatures cannot be used as criteria for identifying proteins.

Levene⁸ in 1899 showed that there is present in brain a protein belonging to the general class of proteins known as nucleoproteins, thought to be derived from the cell nucleus and characterized by the presence of nucleic acid as a prosthetic group. Minced brain was exhaustively extracted with a 4% solution of ammonium chloride. The protein was precipitated from this so-

⁷W. D. Halliburton, J. Physiol., 15, 90 (1894).

⁸P. A. Levene, Arch. Neurol. Psychiatry, 2, 1 (1899).

lution by treatment with dilute acetic acid. The precipitated protein was soluble in an excess of acetic acid and in dilute solutions of alkalies. It contained 0.55-0.57% phosphorus. The solubility was greatly reduced if the protein remained in acid solution for a considerable period of time or if it was dissolved and reprecipitated several times. The protein which had been once dissolved and reprecipitated was extracted with alcohol and ether to remove the lipoids. Of this process Levene states that it was impossible, even after continuous extraction for several weeks, to obtain alcohol and ether extracts which left no residue upon evaporation. Guanine and adenine were identified in the hydrolysate resulting from heating the protein for 10 hours with 2% sulfuric acid. From the products of alkaline hydrolysis, Levene isolated a substance which contained 3.35% phosphorus and which in solution resembled nucleic acid in its ability to precipitate albumoses and proteins from solution. The residue of brain tissue which had been extracted with ammonium chloride was next extracted with dilute alkali to determine whether another protein of different composition could be obtained from it. Considerable protein was removed with 0.5% ammonium hydroxide solution. This protein had the same phosphorus content, gave the same products of hydrolysis and enzymic digestion as the protein from the ammonium chloride extract, leading him to believe that the brain contains only one nucleoprotein.

No other investigations of the proteins were reported until those of McGregor⁹ who, in 1916, made an extensive study of the solubilities of the brain proteins in water and in solutions of various neutral salts. The lipoids were extracted from fresh, minced or air-dried tissue by a solution of 5% absolute ethyl alcohol in benzene, a mixture which he found to be the most effective solvent for the lipoids of the brain. The dried, lipid-free powder was extracted with water or with dilute solutions of neutral salts. The extracts contained a nucleoprotein which was precipitated with acetic acid. Some of the protein remained in solution in amounts which varied in the different extracts. The phosphorus content of the protein was 0.11%. When solutions containing the nucleoproteins were treated with sodium or ammonium sulfates, the amount of protein precipitated increased with in-

⁹H. H. McGregor, J. Biol. Chem. **28**, 403 (1917).

creasing concentration of the salts. This observation and the fact that the protein was continuously precipitated by heat as the temperature was raised led McGregor to conclude that only one protein was extracted by neutral solvents. After the tissue was thoroughly extracted with the neutral solvents, another protein was obtained by treatment of the residue with 1% ammonium hydroxide or with 0.5% sodium hydroxide. This protein was completely precipitated from solution by acetic acid only when the concentration of acid reached 0.1%. It was less easily denatured by acids than the water-soluble protein. After purification, its phosphorus content was 0.6%. Proteins could also be extracted by acidic solvents, as 1% acetic acid or dilute solutions of acid salts, but these proteins appeared to be decomposition products, probably acid metaproteins. McGregor's work thus showed that at least two soluble proteins could be extracted from brain tissue.

During the last few years, R. J. Block¹⁰ and his collaborators have reported a series of investigations on the composition of various classes of proteins in an endeavor to find a basis for the classification of proteins which would be more rational than that based upon their solubilities in salt solutions. It has been recognized for some time that in the serum proteins, for example, the fractions which have been isolated by the widely used procedures of salting out are the "accidental products of the methods employed." Sørensen¹¹ showed in 1930 that crystalline serum albumin is readily and reversibly dissociated into components of different chemical and physical properties. Representing the definite components, i.e., compounds of polypeptides, as A, B, C... and the subscripts by x, y, z... , the molecular complex may be $A_x B_y C_z \dots$. While the constituents of A, B and C are held together in a firm chemical union, the components A, B and C, are probably held together in the complex by weaker secondary valences. It would be expected that by physico-chemical methods of treatment, the latter forces could be overcome and the composition of the resulting products would vary with the method used.

The results of Block's experiments have substantiated this theory. The serum proteins were fractionated by different methods

¹⁰R. J. Block and H. B. Vickery, J. Biol. Chem., **93**, 113 (1931); **103**, 261 (1933); **104**, 347 (1934); **105**, 455 (1934).

¹¹S. P. L. Sørensen, Compt. rend. trav. lab. Carlsberg, **18**, 1 (1930).

of salting out, and the histidine, arginine and lysine contents of the proteins isolated were determined. The proteins obtained did not show a constant composition as regards these acids. The fractions which contained the larger amounts of lysine were the least readily precipitated by neutral salts and appeared to comprise the more soluble albumin fractions with which the components low in lysine might unite to form the globulins. Therefore, since the fractions isolated do not represent chemical entities preexistent in the serum, proteins precipitated by one method should not be compared with those precipitated by another.

Gortner, Hoffman and Sinclair¹² found a similar relationship in the globulins of wheat flour. They found that the solubility of the extractable proteins depends upon both the anions and the cations and upon the concentration of the salts used. At a constant hydrogen ion concentration, the peptizing power of alkali halides decreased with increasing concentration of the salts and that of the alkaline earth salts increased. The peptizing power of anions increased in the order, $F < SO_4 < Cl < Tartrate < Br < I$ and that of the cations in the order $Na < K < Li < Ba < Sr < Mg < Ca$. When normal solutions of the alkali halides were used as solvents, quantities of protein were extracted which varied from 13% for potassium fluoride to 64% for potassium iodide. They concluded that the use of a certain salt in a given concentration cannot give rise to a chemical entity which can be defined as a globulin and that the generalization that a "globulin is a protein soluble in dilute solutions of salts of strong acids and strong bases" does not hold.

Block and Vickery believe that many proteins are characterized by the amounts of the basic amino acid, arginine, histidine and lysine which they contain. In 1931 they reported that keratins from various sources were characterized by a constant proportion of these acids and proposed the following definition: "A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute alkalis and acids, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine and arginine that the molecular ratios are respectively approximately as 1 : 4 : 12." A keratin prepared by them from brain by subsection of the protein

¹²R. A. Gortner, W. F. Hoffman and W. B. Sinclair, Kolloid Z., **44**, 97 (1928).

to the action of pepsin and trypsin contained histidine, arginine and lysine in a molecular ratio of 1 : 2 : 2. Neurokeratin, they claim, is not a true keratin, but is possibly a product resulting from changes in the protein which occur during its isolation. It is also possible that it is another protein which is resistant to digestion by enzymes or a structural protein of small peptizing capacity.

The most recent studies of the soluble proteins of the brain are those of Block and Brand¹³ and appeared after the present investigation was begun. Finely ground brain tissue was extracted with acetone until it was quite dry. The residue was washed with ether and then extracted at 40° with a mixture of 95 parts of benzene and 5 parts of absolute ethyl alcohol. The lipid-free residue represented about 9% of the fresh brain and contained 12% nitrogen.

The soluble protein was extracted from the lipid-free residue by trituration in a mortar with three times its volume of 6.5 M glycerol. This solvent was selected since preliminary tests with various solvents indicated that losses due to denaturation were less than with saline solvents. The clear, glycerol solution was diluted and poured into acetone at 0° The flocculent precipitate was washed with acetone and ether and dried. The dry protein represented 12-13% of the lipid-free residue and contained 17.3% nitrogen.

When glycerol was used as the solvent it seemed to be equally satisfactory to extract the fresh brain directly without previous extractions with lipid solvents. The ground tissue was placed in sufficient absolute glycerol to make a final concentration of not less than 45% by volume. The suspension was covered by a layer of toluene and the extraction allowed to continue for about 5 days with shaking. The resulting pulp was then diluted with an equal volume of water and filtered. The residue was extracted again with 25% glycerol. The filtrates were combined and poured into 8 to 10 volumes of cold acetone¹⁴. The precipitate

¹³R. J. Block and E. Brand, Psychiatric Quarterly, 7, 613 (1933).

¹⁴It would seem that by such treatment the cerebrosides might be extracted and carried down with the protein. Since cerebrosides can readily be brought into colloidal solution in water, glycerol would also be expected to be a good peptizing agent. Any cerebrosides extracted by glycerol would then be precipitated by acetone, cerebrin, especially, being quite insoluble in this solvent.

was washed with acetone and ether. The yield was about 3-4% of the moist brain or 50% of the total protein.

The dried proteins thus obtained were extracted with ice water containing a little acetic acid. A soluble fraction, assumed to be albumin and which comprised about 5% of the dry protein, was obtained by this procedure. The residue was extracted with a 10% solution of sodium chloride. The globulin extracted amounted to only about 1% of the dry protein. The residue from the two extractions was completely soluble in either 1.0 N ammonium hydroxide or 0.1 N sodium hydroxide and could be precipitated from the solutions by the addition of acetic acid. This fraction, the "nucleoprotein," represented about 90% of the dry protein. The protein gave positive tests for cystine (labile sulfur) and for tyrosine. No analyses of the amino acid content were reported nor was the protein not extractable by glycerol investigated.

PART II

METHODS OF ISOLATION AND THE PROPERTIES OF
THE PROTEINS OF BEEF BRAIN

The investigation reported here was undertaken as a part of the general program for the study of the chemistry and pathology of the brain, as being carried out in the Otho S. A. Sprague Memorial Institute. The object of this study was to devise a method for the isolation of the brain proteins and to study their chemical and immunological properties.

Since the older, empirical methods of separating proteins by means of their solubilities in salt solutions have not been found to be satisfactory, it was decided to base the study upon the colloidal behavior of the brain substance as a whole. An extremely stable sol is formed when the finely ground tissue is stirred into a large volume of water and the coarser particles are allowed to settle out. When the pH of the sol is adjusted to about 4.6 by the addition of hydrochloric acid, a flocculent precipitate is formed. The water-clear supernatant liquid contains the water-soluble extractives¹⁵ and only traces of the proteins and lipoids. The precipitate is composed of protein and lipid in the combination as they exist in the brain. The exact nature of the union is not known. It may be a weak, chemical union in which the protein and lipid are held together in a complex, known as a coacervate, formed by the electrostatic attraction between colloidal particles of opposite charge. Adsorption valences might also act as the binding force between the components of the complex. The protein-lipoid complex precipitated from the aqueous sol made from the whole brain was used as the starting material for the separation of the lipid from the protein and the subsequent study of the proteins thus isolated.

The sol and the precipitate of the complex were prepared as follows:

The outer membranes with the adhering blood vessels were

¹⁵Cf. W. Koch, *Am. J. Physiol.*, 11, 303 (1904), for a discussion of these materials and their composition.

removed from fresh beef brains. The brains were washed for a short period in cold, running water and then ground with a meat grinder. The ground tissue was frozen in an ice-box at $-20 \pm 5^{\circ}$. If the tissue, while still solid, was ground again in a chilled grinder, a fine, homogeneous mixture was obtained. After the grinding, the material, in a semisolid form, was added slowly, with mechanical stirring, to a large volume of distilled water. The proportion found to be most satisfactory was about 500 g. of the moist brain to 14 l. of water. The emulsion was allowed to stand overnight in an ordinary ice-box. The supernatant liquid was siphoned from the coarser particles which had settled. The sediment was emulsified and allowed to settle again. After two or three such treatments all of the readily emulsifiable material was removed and the final sediment, which appeared to consist of connective tissue and the coarser particles of white matter, was discarded.

The sol thus formed showed little tendency to settle, even if kept for several days. Hydrochloric acid (1.0 N) was added to the sol until precipitation was complete. This occurred at about pH 4.6. After the precipitate had settled, the clear supernatant liquid was decanted and the precipitate was collected by centrifugation. If the precipitate could not be used immediately, it was kept in the frozen state at -20° . The material thus prepared was used for determining the best methods for the separation of the lipoids from the protein and for further study of the isolated protein.

Two procedures will be described for the separation of the proteins and lipoids. The first (A) consists in the treatment of the frozen precipitate from the brain sol with acetone or alcohol and ether in the course of which the precipitate is dehydrated and some of the lipoids are removed. To complete the separation, the dry, partially extracted protein is extracted exhaustively with chloroform in a Soxhlet apparatus. The second method (B) consists in treating the precipitate from the brain sol in such a manner as to reverse the phase with the result that the system resembles a water-in-oil rather than an oil-in-water dispersion.

The properties of the proteins prepared by each of the two methods will be described separately. In addition to the method of obtaining the proteins from the isoelectric precipitate of the brain sol, a method will be described in which the brain sol is

precipitated by metaphosphoric acid and the precipitate obtained treated according to method B for the removal of the lipoids.

The complete separation of the lipoids from the protein without causing changes in the nature of the protein is difficult. The greater part of the lipoids can be removed simply by shaking the moist, finely divided tissue with solvents for the lipoids under such conditions that the particles of the sol are wetted by the solvent used in the extraction. However, the removal of the last traces of the lipoids requires more drastic treatment. While the separation is much more effective if heat can be applied, as is customary when the lipoids only are to be used, subjection of the protein to heat must be avoided as much as possible.

Method A

An attempt was made to avoid changes in the protein by carrying out the extraction at very low temperatures. This, alone, was found to be ineffective, even if the period of extraction was long. When followed by extraction at the temperatures reached in extraction by means of a Soxhlet apparatus, a very good separation was made. This procedure had the advantage that the protein was not subjected to exhaustive treatment with lipid solvents at temperatures above freezing until all of the water had been removed.

The presence of lipoids of different kinds requires that a series of solvents be used. Benzene or chloroform are effective as solvents for cholesterol, lecithin and kephalin while the alcohols are better solvents for the cerebrosides, which contain a larger number of polar groups. Accordingly, chloroform was used as the solvent in the extractions made after the removal of water with acetone or alcohol at -20° .

To test the effectiveness of extraction at -20° , the following experiments were carried out. The collected precipitate, prepared as described above, was frozen in the ice-box kept at -20° . While the precipitate was still at this temperature, it was treated with two to three volumes of acetone or 95% ethyl alcohol. When the solvent became diluted by the water absorbed from the precipitate, it was poured off and replaced continuously until the brain substance was completely dehydrated. The dry substance was then extracted twice with absolute alcohol and once

with ether, still keeping it at the low temperature. The ether was poured off and the last traces of the solvents were removed from the residue, by drying over calcium chloride in a vacuum desiccator.

To determine how much extractable lipid was still present, a sample of 8.25 g. of the dry powder was placed in the extraction thimble of a Soxhlet apparatus and extracted with chloroform until no more lipid could be removed. In this process, a total of 3.31 g. of lipid was extracted, which represented 40% of the dry weight of the partially extracted precipitate. This result showed that the treatment with alcohol and ether at a low temperature removed only a small fraction of the lipids.

Since a large portion of the lipid seemed to be soluble in chloroform, but insoluble in alcohol and ether, the extraction at a low temperature was repeated, using chloroform in addition to alcohol and ether. Another portion of the frozen precipitate was treated with 95% alcohol until the protein was dehydrated. It was then subjected to six extractions with chloroform at the same temperature. From 1.912 g. of the material treated with alcohol at -20° , only 0.302 g. of lipid was removed by the six extractions with chloroform. This represented 15.3% of the dry weight, whereas extraction by chloroform in a Soxhlet apparatus in the first experiment had removed 40% of the dry weight of the protein which had been treated in the same way with alcohol. It was concluded that extraction with different solvents at temperatures above freezing was necessary.

These results led to the adoption of the first procedure which consisted in (a) dehydration of the precipitated protein-lipoid complex at -20° , (b) partial extraction of lipoids, at the same temperature, with alcohol and ether and (c) a final extraction in a Soxhlet apparatus with chloroform until no more lipid could be removed. Extraction at -20° with chloroform was omitted since only relatively small quantities of lipid were removed. The lipid extracted in the final step, (c), amounted to approximately 40% of the dry weight of the precipitate extracted at -20° . The completely extracted and dried preparations contained 0.41-0.71% phosphorus and 12-13% nitrogen.

Analysis and Properties of the Protein Isolated by Method A

The protein, which was now as free of lipoid as was possible to obtain it by the method used, was only partially soluble in dilute acids and alkali. It was found that at given hydrogen ion concentrations only certain fractions of the protein were soluble and that the various fractions were characterized by different contents of phosphorus. Determinations of the solubility were carried out as follows:

I. A sample of 0.846 g. of the dry protein was ground with 0.1 *N* hydrochloric acid and, after it had stood for 1.5 hours, was centrifuged. Some of the protein was peptized forming a slightly turbid solution. Upon addition of sodium hydroxide no isoelectric precipitate could be obtained. To recover the protein, the solution was evaporated to dryness on the water bath, the residue was washed with alcohol and ether and dried in vacuo. The undissolved protein remaining after the extraction with acid was next shaken with 0.01 *N* sodium hydroxide. The mixture was centrifuged and a clear alkaline solution separated from an insoluble residue. The protein in the alkaline solution was completely precipitated by adjusting the pH to 5.6-5.8.¹⁶ The insoluble residue, which had become swollen by the treatment with alkali, was dehydrated by the addition of a little acid. The precipitated, soluble protein and the insoluble residue were washed with alcohol and ether and dried in vacuo. The results of the fractionation are included in Table I.

II. A sample of 0.950 g. of the dry protein was ground with 0.1 *N* hydrochloric acid. The acid-soluble portion was separated from the remaining protein by centrifugation and decantation. The acid-soluble protein was precipitated from the solution by adding sufficient sodium solution to adjust the pH to its isoelectric point. The precipitate was collected, washed with alcohol and ether and dried in vacuo. The supernatant liquid obtained from the precipitation was evaporated to dryness on the water bath and the residue, or water-soluble fraction, was washed

¹⁶The pH values mentioned here and in the experiments described hereafter, unless otherwise specified, were determined by the spot method with indicators. Later measurements made with a glass electrode indicated that these values may have been somewhat too high.

and dried. The material remaining after the treatment with acid was shaken with 0.01 N sodium hydroxide solution and an alkali-soluble fraction and insoluble residue were obtained as in the previous experiment. The results are summarized with those of experiment I in Table I.

TABLE I

Preparation	Weight in mg.		Distribution of recovered protein (%)		Phosphorus content (percent)		Distribution of recovered phosphorus	
	I	II	I	II	I	II	I	II
Original material	846	950			.68	.71		
Acid-sol. fraction	16*	38		5.9		.33		4.2
Water-sol. fraction		13	2.8	2.1	.78	.10	4.6	...
Alkali-sol. fraction	362	282	63.3	44.5	.52	.60	70.8	55.8
Insol. residue	194	301	33.9	47.5	.34	.40	25.0	39.5

*In I, the acid-soluble and water-soluble fractions were not separated.

The composition and amount of each fraction varied with only slightly different experimental conditions. About 67% of the original dry protein was recovered in the fractionation procedures. In Experiment I, 63.3% of the recovered protein was in an alkali-soluble form and had a phosphorus content of 0.52%. In Experiment II, 44.5% of the recovered protein was in this form and contained 0.60% phosphorus. The fractions soluble at the lower pH values had a higher concentration of phosphorus than the less soluble forms. This relation has been observed in all of the succeeding experiments. The peptizability of the protein apparently depends upon the volume of the solution used and upon various physical factors, such as the period of contact with alkali and thoroughness of mixing, as well as upon the pH at which the solution is made.

The following experiment was carried out to determine the relative hydrogen ion concentrations at which the whole protein showed the highest solubility. A sample of 2.002 g. was suspended in water and thoroughly extracted at pH 3.0-3.5. The protein soluble at this pH was collected and dried. The extraction was followed by others at pH 7.0-7.4, 8.0-8.2, 8.5, and 9.2. The mate-

rial remaining after these extractions were made was then treated successively with 0.01 N, 0.1 N and 0.2 N sodium hydroxide. The amounts of protein obtained in each extract are tabulated below.

TABLE II

Conditions of Extraction	Amount of Protein Dissolved, g.
Water-soluble*	0.010
pH 3.0-3.5	.037
pH 7.0-7.4	.090
pH 8.0-8.2	.040
pH 8.5	.018
pH 9.2	trace
with 0.01 <u>N</u> NaOH	.044
with 0.1 <u>N</u> NaOH	.216
with 0.2 <u>N</u> NaOH	.133
insol. residue	0.500

*Recovered from the supernatant liquid obtained when the extract made at pH 3.0-3.5 was precipitated.

The results showed that a maximum amount of soluble protein was extracted at pH 7.0-7.4 and that little more was removed by slight increases in alkalinity. With 0.1 N sodium hydroxide another large fraction was extracted. About 25% of the original protein was insoluble in 0.2% alkali. Because of the danger of hydrolysis it was thought not to be advisable to use higher concentrations of alkali.

On the basis of this experiment it was decided to make an arbitrary division of the protein into three fractions, one comprising all of the material soluble up to pH 8.5, the second composed of the material which could then be extracted with 0.1 N sodium hydroxide and the third, the remaining material which was insoluble under these conditions.

The preceding experiments indicated that the whole protein is a complex mixture made up of components ranging from those readily peptized in acid, neutral or weakly alkaline solution to those requiring stronger alkali for their peptization and including those insoluble in alkali or requiring for their solution alkali of such strength as would cause their denaturation or destruction of the protein. Fractions extracted at about the same pH would be expected to be fairly similar in their properties and composition and markedly different from those extractable at a very different pH. To test the validity of this assumption it was

necessary to make comparative analyses of the three fractions.

Accordingly, sufficient quantities of each of the three fractions were prepared to make possible the determination of the distribution of amino acid nitrogen in each fraction and the total contents of nitrogen and phosphorus. The lipoids were removed from the isoelectric precipitate of the brain sol as described in the preceding section (p. 9). The following experiment is described in detail to illustrate the procedure adopted for the preparation of the three fractions.

A sample of whole, lipid-free protein, 12.045 g., was ground with water which was added a few drops at a time until the powder was thoroughly moistened. The material was transferred to a two-liter bottle and water was added to make a total volume of about 800 cc. Approximately 40 cc. of 0.1 N sodium hydroxide solution was required to adjust the pH of the mixture to 8.2-8.4. The mixture was shaken for one hour in a mechanical shaker. It was centrifuged, the supernatant liquid was decanted and the residue returned to the bottle. A second extraction was made at the same pH. The two extracts were combined and treated with sufficient 0.1 N hydrochloric acid to cause the maximum precipitation. The precipitate was collected, washed with alcohol and ether and dried in vacuo. The material, designated as Fraction I, weighed 2.553 g. and represented 21.2% of the original sample.

The residue which remained after the two extractions at pH 8.2-8.4 was returned to the bottle. About 1500 cc. of 0.1 N sodium hydroxide was added and the mixture was shaken for one hour. The material in solution was separated by centrifugation and the residue treated a second time in the same manner. The two extracts were combined and precipitated by the addition of sufficient 1.0 N hydrochloric acid to adjust the solution to its isoelectric point. It was found that a large portion of this fraction could now be dissolved at pH 8.0-8.2. The moist precipitate was suspended in a total volume of 1500 cc. of water, adjusted to pH 8.2 and shaken for one hour. The protein which dissolved was separated by centrifugation from a smaller portion which did not dissolve at this pH. The protein in solution was precipitated isoelectrically and dried. This constituted Fraction II and weighed 3.752 g. The smaller part was dissolved in 0.1 N sodium hydroxide and precipitated isoelectrically. When dry, it weighed 0.40 g. and was designated as Fraction II-a.

The material left after the two extractions with 0.1 N alkali was extracted a third time with alkali of the same strength. Since this extract contained very little protein no further alkaline extractions were made.

The material undissolved after the treatment with alkali was suspended in water and sufficient 0.1 N hydrochloric acid was added to adjust the protein to its isoelectric point. The flocculated protein was collected, washed and dried. The dry material weighed 1.796 g. and constituted Fraction III.

The results of this fractionation and two others carried out in the same manner are summarized in Table III.

TABLE III

	Fractionation a		Fractionation b		Fractionation c	
	Wt. in g.	Percent Recovered	Wt. in g.	Percent Recovered	Wt. in g.	Percent Recovered
Whole protein	12.045		10.326		7.037	
Fraction I	2.553	21.2	1.883	18.2	1.449	20.6
Fraction II	3.752	31.2	3.871	37.5	2.876	40.8
Fraction II-a	.400	3.3	.202	1.95	.171	2.4
Fraction III	1.796	14.9	1.188	11.5	.67	9.6

It is seen from the results that the relative amount of protein in each fraction varied with approximately identical experimental conditions. From 18 to 21% of the whole protein was soluble below pH 8.4. A greater variation occurred in Fraction II, where the amount soluble in 0.1 N alkali ranged from 9.5 to 15%. The total recovery of protein varied from 69 to 73% in the three fractionations. There were large, unavoidable mechanical losses due to the large number of manipulations involved. The supernatant liquids decanted from the isoelectric precipitates contained some protein. It was difficult to adjust the acidity to the exact isoelectric point in each case. To avoid denaturation as much as possible, it seemed more desirable to carry out the entire procedure as rapidly as possible than to try to obtain quantitative recoveries. While the results may not represent the absolute amounts soluble under the given conditions, they are of value for comparison.

The composition of the three fractions was investigated next. The amino nitrogen partition was carried out according to

the method described by Van Slyke.¹⁷ The modified procedure¹⁸ was used for the decomposition of the phosphotungstic acid precipitate of the basic amino acids by extraction with a mixture of amyl alcohol and ether. The nitrogen determinations were made by the semimicro Kjeldahl method of Hitchcock and Belden.¹⁹ The phosphorus contents were determined by the colorimetric method of Fiske and Subbarow.²⁰

Fraction II-a was analyzed in one instance, but, owing to the small quantities available, a complete analysis of the other samples was not made.

The results of the analyses are summarized in Table IV.

TABLE IV
COMPOSITION OF BRAIN PROTEIN FRACTIONS

Form of Nitrogen, as Percentage of Total Nitrogen	Fraction I		Fraction II		Fraction II-a	Fraction III	
	Experiment A	Experiment B	Experiment A	Experiment B	Experiment A	Experiment A	Experiment B
Ammonia N	8.43	8.15	7.63		7.33	6.81	6.03
Melanin N	3.65	3.40	2.19	2.49	2.94	4.54	4.88
Basic Acids							
Total N	24.28	22.26	27.30	22.64	25.79	24.49	20.25
Amino N	11.31	11.46	14.64	12.61	12.45	12.76	12.23
Non-amino N	12.97	10.80	12.66	10.03	13.34	11.73	8.02
Arginine N		11.00	12.51	12.70	12.00	6.47	9.51
Histidine N		4.04	4.85	0.75	6.43	10.64	1.31
Cystine N							
Lysine N		7.29	5.77	9.22	7.33	6.09	9.42
Filtrate from bases							
Total N	63.47	65.40	63.81	68.37	65.07	64.04	71.95
Amino N	53.70	64.21	61.84	62.36	59.54	61.29	66.10
Non-amino N	9.77	1.19	1.97	6.01	5.53	3.65	5.85
Total nitrogen (percent by wt.)	15.16	15.55	15.83	15.56	16.14	13.87	14.49
Total phosphorus (av. of several)		0.57		0.19			0.18

¹⁷D. D. Van Slyke, J. Biol. Chem., 10, 15 (1911); 12, 275 (1912); 16, 121 (1913).

¹⁸Ibid., 22, 281 (1915).

¹⁹D. I. Hitchcock and R. C. Belden, Ind. Eng. Chem. Anal. Ed., 5, 402 (1933).

²⁰C. H. Fiske and Y. Subbarow, J. Biol. Chem., 66, 375 (1925).

Experiments A and B represent two different fractionations carried out in the procedure described above. The results of the amino nitrogen distribution are expressed in percentage of total nitrogen in the hydrolyzed sample of protein.

The above results confirm the view that the protein is a complex mixture and that the various fractions isolated do not represent chemical entities. It can be seen that the same fractions prepared at different times do not have a constant composition. The variations shown are greater than can be accounted for by experimental error in the analysis.

A comparison of the three fractions shows that as far as the amino acid nitrogen distribution is concerned, there are no pronounced differences. Fraction III shows a somewhat higher content of melanin nitrogen and lower percentages of total nitrogen than the other two fractions. The concentration of cystine was very small. For an accurate determination of the concentration a larger sample of protein would be required than could be handled conveniently by the Van Slyke procedure. A few estimations were made by the colorimetric method of Folin and Looney.²¹ The results indicated that the amount of cystine nitrogen was not greater than 0.2% of the total nitrogen although the determinations were not entirely satisfactory because of the brown color of the hydrolyzed protein solutions. The decrease in phosphorus content as the solubility of the protein fractions decreases has been noted throughout. The significance of this variation will be discussed later in connection with results of other experiments.

Method B

Although the protein used in all of the preceding experiments had been thoroughly extracted with lipid solvents, further experiments indicated that appreciable amounts of lipoidal material were still present. In the course of the fractionations, it was noted that the alcohol washings of the moist, freshly precipitated protein, which had just been extracted with alkali, were often slightly colored. When the alcohol was evaporated, a small residue was left which was soluble in lipid solvents. The treatment with alkali had apparently made the lipid remaining more

²¹O. Folin and J. M. Looney, J. Biol. Chem., 51, 421 (1922).

available for the lipid solvents, presumably by breaking up the protein membranes.

When the protein was subjected to peptic digestion at 37°, a larger amount of lipid was set free and could easily be extracted from the products of hydrolysis. A sample of 5.085 g. of the whole, unfractionated protein was digested with pepsin until the digest gave a negative biuret reaction. The portion of the hydrolyzed product which was soluble at pH 9.0 could be precipitated at pH 2.0. When this precipitate was collected and washed with alcohol and ether, the washings were found to contain material of lipoidal nature, which was recovered by evaporation of the solvents. The high phosphorus content of the residue, 1.97%, and its solubility in various organic solvents indicated that it contained phospholipides.

In view of these observations it seemed necessary to devise a more effective method for removing the lipoids. It had been noted that when the moist, isoelectric precipitate obtained from the brain sol was treated with small amounts of benzene or toluene, a thick paste of putty-like consistency was formed. This could be worked with a spatula and a large part of the water present could be removed as in working butter. A reversal of phase had occurred in which the solid phase of high lipid content instead of the water had become the continuous phase. Thus it would be expected that lipid solvents would have a better opportunity to come into intimate contact with the lipoids and an efficient extraction could be made by extracting with a series of solvents ranging from the non-polar, e.g. benzene, toward the polar, e.g. methyl alcohol, type of lipid solvent. This method was tested and found to be satisfactory. The following procedure was adopted:

The isoelectric precipitate of the brain sol was prepared as described on page 9. Benzene, a few drops at a time, was stirred into small portions of the precipitate (about 50 g.) until the phase was inverted. The paste formed was worked with a spatula until the greater part of the water was removed. Any excess benzene was removed with the water. The paste was transferred to a three-liter bottle and shaken with about two liters of chloroform for one hour. A sol was obtained with the lipid solvent the continuous phase. The mixture was allowed to stand over night at room temperature, then centrifuged and the chloroform drawn off with gentle suction from underneath the layer of protein. Prelim-

inary experiments had indicated that two extractions carried out in this manner removed 90% or more of the material extractable with chloroform at ordinary temperatures. At the end of two extractions, the material contained a very small quantity of water. It was next extracted with 95% ethyl alcohol. Sufficient alcohol to make a final concentration of 85% or more was added quickly, since under these conditions denaturation by alcohol is supposedly avoided.²² Three or four extractions with ethyl alcohol were made in the same manner as with chloroform and these were followed by two or three extractions with ether.

At this stage the extracted protein was dried in a vacuum desiccator and ground in a mortar. The powder was sifted through a 60-mesh wire gauze and a small quantity of coarser particles was discarded. From 20 beef brains 235 g. of the fine, dry powder was obtained.

To complete the extraction, the dry powder was next extracted in a Soxhlet apparatus with chloroform, methyl alcohol and ether in succession, the ether being used to remove the alcohol. The first extraction with chloroform, which was continued for 20 to 24 hours removed an amount of lipid representing 0.3-0.6% of the dry powder. A second extraction removed only 0.04-0.1%, showing that the wet extraction with chloroform had been almost complete. The amounts removed by methyl alcohol varied widely in each lot extracted. The protein showed a marked tendency to swell when in contact with methyl alcohol. The swelling in a partially filled thimble was often sufficient to burst the thimble. The amounts removed by each extraction expressed in percentage of the dry weight before extraction with methyl alcohol are summarized in Table V. Extractions were continued until very little

TABLE V
EXTRACTION OF PROTEIN WITH METHYL ALCOHOL

Extraction	Amount of Lipoid Removed (Percentage of Dry Weight)
1st	1.6 - 3.7
2nd	0.6 - 2.5
3rd	0.3 - 1.3
4th	0.2 - 1.3
5th	0.2 - 0.7

²²W. Koch, J. Am. Chem. Soc., 31, 1229 (1909).

material was removed. This required five extractions of 17 to 20 hours each. The protein was ground and dried in vacuo between the extractions.

After the extraction with methyl alcohol, the dry protein was extracted with ether. Only traces of a lipid-like substance were removed. The extraction, however, appeared to improve the physical condition of the protein, leaving it in a more finely divided form.

The method used for extracting the lipoids in the earlier experiments involved wet extractions with ethyl alcohol at a low temperature and extraction with chloroform only in the Soxhlet apparatus. In the second method chloroform was used for the first wet extraction and was followed by extraction with ethyl alcohol after most of the water had been removed. No methyl alcohol was used in the first method. This appears to have a bearing upon the marked differences in solubility shown by the new protein. The second preparation also had a much lower phosphorus content than the protein analyzed previously.

Analysis and Properties of the Protein Isolated by Method B

The solubility of the new preparation was studied by separating it into the three somewhat arbitrarily chosen fractions as before. Because of the low solubility of the new protein at pH 8.2, a pH of 9.0 was chosen as the upper limit of Fraction I. Fraction II consisted of the protein soluble in 0.1 N alkali and in this case it was not dissolved and reprecipitated. The results of the fractionation are given in Table VI together with the corresponding values for the first preparation for the purpose of comparison.

It appears from a comparison of the two preparations that in the attempt to remove the last traces of lipid, the protein has been rendered less soluble. Part of the material which in the earlier preparation would have been in Fraction II now appears in Fraction III and part of Fraction I now appears in II. The action of methyl alcohol on the protein observed during the extraction as noted above and the fact that it was not used in the first preparation suggest that this reagent may be responsible for the change. Some group of the cerebrosides may be a peptizing agent for the protein and may have been removed by the methyl al-

TABLE VI

Preparation	Percentage of Original Protein in the Fraction	Phosphorus Content (percent)
Whole protein		
Method A		0.52
Method B		0.33
Fraction I		
Method A	1.82 - 20.6	0.57
Method B	3.2 - 3.7	1.2 - 1.7
Fraction II		
Method A	31.2 - 40.8	0.19
Method B	25.9 - 27.2	0.15
Fraction III		
Method A	9.6 - 14.9	0.13
Method B	58.5 - 61.8	0.05

cohol. Another possibility is that denaturation changes may have taken place during the wet extraction. The extraction of the large quantities of protein and removal of the solvent by centrifugation necessarily required that the protein remain in contact with the solvents for long periods of time.

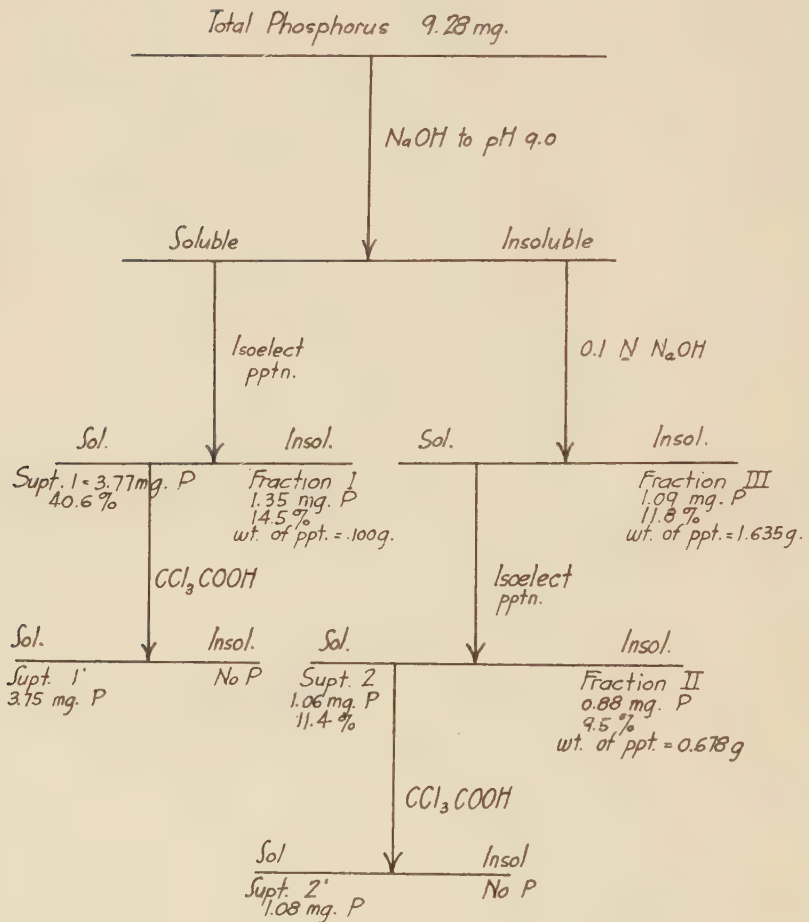
The phosphorus contents of the Fractions II and III were remarkably constant (the figures given represent the average of seven fractionations). That of Fraction I was higher than in any protein fraction previously obtained and was more variable.

The high phosphorus content of the more soluble fractions suggests at once the possibility that they contain a larger proportion of nucleic acid or other phosphorus containing substance of acidic nature than the less soluble fractions. Studies of the distribution of phosphorus in the various fractions and supernatant liquids showed that, in the fractionation procedures, phosphorus was split off in an acid-soluble but nonprotein form. When the alkaline solutions of the soluble fractions are precipitated by the addition of acid, a large part of the phosphorus which was originally present remains in the solutions decanted when the precipitates are collected.

The results of one of the experiments in which the distribution of phosphorus was determined are summarized diagrammatically in Chart I. A sample of 2.8086 g. of protein with a total phos-

CHART I

SCHEME OF FRACTIONATION AND DISTRIBUTION OF PHOSPHORUS



phorus content of 9.28 mg. was used for the fractionation. The fractions were prepared as described on page 16. The clear, supernatant liquids, designated as Supts. 1 and 2, which were decanted from the isoelectric precipitates, were concentrated by evaporation on a water bath and made up to a volume of 100 cc. Aliquots of the concentrated solutions were used for the determination of phosphorus. For the determination of the concentration of phosphorus in the protein precipitable by trichloroacetic acid, other aliquots were treated with sufficient trichloroacetic acid to make a final concentration of 10%. The protein precipitated was collected by centrifugation, the supernatant liquids were decanted and made up to a known volume (designated as Supts. 1' and 2'). The figures in parentheses give the percentage of the total phosphorus found in each of the fractions and the weights of the precipitates are given wherever possible. The supernatant liquid obtained in the preparation of Fraction I in this experiment contained 40.6% of the total phosphorus. This proportion varied from 33 to 40% in other experiments. Supernatants obtained by the precipitation of Fraction II contained from 11 to 18% of the total phosphorus. The seemingly high percentage, 11.6%, recovered in Fraction III, which has the very low phosphorus content of 0.05%, is accounted for by the fact that this fraction represents 58 to 62% of the total weight of the whole protein (cf. Table VI).

Although these supernatants contained small amounts of protein which was precipitable by trichloroacetic acid, no phosphorus was bound to the protein so precipitated. The phosphorus in solution was thus in an acid-soluble but nonprotein combination. Whether the phosphorus-containing substance in the supernatant was nucleic acid could not be determined since the amount present was too small to isolate.

To some extent, trichloroacetic acid can replace the nucleic acid or other phosphorus containing substance of the protein fractions. When a portion of Fraction II was extracted several times with a 10% solution of trichloroacetic acid, about 28% of its phosphorus was extracted. When Fraction III was treated in the same way, about 5% of its phosphorus was removed.

The phosphorus content of the protein of Fraction I became higher when the protein was dissolved in alkali and reprecipitated. This was illustrated in an experiment in which the phosphorus content of a portion of this protein was increased from its

original content of 1.56% to 1.83% when it was redissolved and reprecipitated twice. At the isoelectric point of the protein, the phosphorus apparently is not removed. When the protein is treated with alkali, keeping the pH below 9.0 but still above the isoelectric point, the more soluble parts of the complex are split off, leaving the less soluble part undissolved. In solution above the isoelectric point of the complex, the protein and nucleic acid are probably largely dissociated and since the nucleic acid is soluble at this pH, it is concentrated in the soluble fraction when it is flocculated with acid. Therefore, the nucleic content of this fraction is higher than that of other fractions. By continuous extraction with alkali, the equilibrium which exists between the nucleic acid in solution and that still combined with the undissolved protein is disturbed. As the soluble parts are removed, more and more of the nucleic can dissociate. Other phosphorus-containing substances of acidic nature which could form complexes with protein would dissociate in the same way. The low content of purine nitrogen in the protein indicates that a large part of the phosphorus of the supernatant liquids is not nucleic acid phosphorus. Theoretically it should be possible to remove all of the phosphorus and obtain a final product containing no phosphorus. While it was not practicable to test the point experimentally, such a condition was approached in the final fractions, which contained a very small and exceptionally constant percentage of phosphorus, 0.04-0.07%. This concentration may represent a minute part not removable under the experimental conditions. Or, since this value was constant in all of the fractions prepared, it is possible that another phosphorus-containing substance may be a fundamental part of the protein molecule, not loosely combined as in the case of nucleic acid.

By using large amounts of material, Levene was able to identify two of the purine bases of nucleic acid in the products of acid hydrolysis. He did not give any quantitative data, but stated that the "cerebronucleoprotein" contained less phosphorus than other true nucleoproteins. The protein prepared by the methods described here appeared to contain a very small proportion of nucleic acid. An attempt was made to determine quantitatively the amount of purine nitrogen present, in order to gain information regarding the proportion of nucleic acid present. The methods usually used involve hydrolysis of the protein with sulfuric acid

and treatment of the hydrolysate with copper sulfate and sodium bisulfite to precipitate the purines. The nitrogen content of the precipitate is then determined by the usual methods. Before the precipitation of the bases is carried out, other products of hydrolysis must be removed. Precipitation of the products with tungstic acid²³ or acetic acid²⁴ or procedures in which the hydrolytic products are held in solution²⁵ have been recommended. If only traces of purines are present in the hydrolysate, it is probable that a significantly large proportion of the purines will be lost in these procedures. The amounts of purine nitrogen found in the precipitated copper compounds from the hydrolyzed brain protein varied widely with the procedures used and often the amounts actually found were not much greater than the blanks involved in the nitrogen determination, itself. No conclusion could be drawn as to the percentage of purine nitrogen present in the brain protein. A qualitative test for the presence of nucleic acid was made by subjecting the protein to the same treatment as that used in isolating thymus nucleic acid from tissues. From 5.0 g. of protein about 2.5 mg. of a crude preparation was obtained which gave the same color reactions when tested with diphenylamine as pure nucleic acid.²⁶

The possibility that nucleic acid was lost during the extraction of the protein-lipoid complex because of a preferential solubility in one of the lipid solvents was considered. In method B, in which the protein had been subjected to long periods of extraction with methyl alcohol, the phosphorus content of the extracted protein was lower than that of the protein prepared by method A where no methyl alcohol had been used. Nucleic acid, alone, was similarly extracted in the same kind of an extraction thimble with methyl alcohol for a long period. The extract contained a very small amount of nucleic acid which could possibly have been carried through the thimble as a fine powder as a result of the long continued extraction. It was concluded that there could have been no loss of nucleic acid due to preferential solution in methyl alcohol and that the lower phosphorus content of

²³G. Schmidt, Z. physiol. Chem., 219, 191 (1933).

²⁴N. Poczka, Deut. Arch. klin. Med., 167, 177 (1930).

²⁵S. Graff and A. Maculla, J. Biol. Chem., 110, 69 (1935).

²⁶Z. Dische, Mikrochemie, 8, 4 (1930).

the second preparation was the result of the more complete removal of phospholipides.

A Comparison of the Proteins of the Brain with the Corresponding Proteins of the Liver

The increased proportion of the insoluble residue, comprising Fraction III found when Method B was used, suggested that this fraction is formed as a result of changes which occur during its preparation. If this is the case, proteins from other organs should yield a similar insoluble residue when they are treated by the same or similar methods. To test this point and to make a comparative study of the other fractions obtained by treatment with alkali in varying concentrations, the proteins of beef liver were investigated. The general method employed was that used for the brain.

An aqueous emulsion of the liver tissue was made and treated with sufficient 0.1 N hydrochloric acid to cause the maximum flocculation. This occurred at about pH 4.6. The supernatant liquid was colored and contained a large amount of soluble protein which was precipitable upon saturation with ammonium sulfate. This was discarded since only the fraction precipitated by acid, the so-called "nucleoprotein," would be comparable to the preparations of brain protein. The precipitated protein, which was colored yellow, was collected by centrifugation and washed repeatedly with distilled water adjusted to the isoelectric point of the protein until the washings were colorless. In this process a large part of the colored substance in the precipitate was removed. Upon treatment of the precipitate with benzene no reversal of phase could be obtained. The extraction with chloroform at this stage was omitted since a solvent which would wet the particles of the sol is required. The precipitate was treated at once with sufficient 95% ethyl alcohol to make a final concentration of alcohol of about 90%. A second extraction with alcohol and two extractions with ether were made. The protein was next extracted in a Soxhlet apparatus with chloroform which removed only a small amount of lipid. Extraction with methyl alcohol was carried out next and, unlike the extraction of the brain protein with this solvent, the extraction was complete in a comparatively short time. Extraction with ether in the Soxhlet removed only traces of lipoidal material. The lipoids appeared to be separated from

the protein with greater ease than from the brain protein. The first extractions of the precipitate had apparently removed the greater part of the lipoids.

The dry, extracted protein was separated into three fractions, corresponding to those made with the brain protein, Fraction I, soluble at pH 8.2-8.6; II, soluble in 0.1 N sodium hydroxide and III, an insoluble residue. The results of the fractionation are summarized in Table VII.

TABLE VII

	Percentage of Whole Protein in the Fraction	Phosphorus Content (Percent)
Whole protein	...	0.56
Fraction I	10.9	2.15
Fraction II	66.7	0.22
Fraction III	6.4	0.05

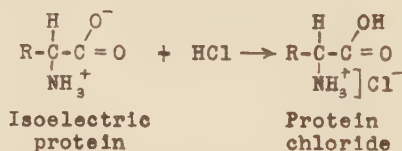
The results show that the solubility of this protein differs markedly from that of the brain protein. A large part of the protein was recovered in the soluble fractions. The insoluble portion may have been an accidental result of the method of preparation. When the protein which had been shaken with 0.1 N sodium hydroxide was centrifuged, a pellicle of apparently insoluble material remained on the surface. By continued treatment with alkali all of the material which could be packed at the bottom of the centrifuge tube was dissolved and the insoluble portion consisted only of the pellicles which had floated on the surface. It is noted also that the phosphorus content of the Fraction I was much higher than that of the corresponding fraction of brain protein. The isoelectric point of this fraction was lower, about pH 3.8 as determined by indicators, while that of Fraction II was much higher, approximately pH 7.0.

The protein of the liver, like that of the brain, appears to be a complex mixture of indefinite composition. Its more soluble components may be more acidic and contain a larger proportion of nucleic acid than those of the brain protein. This is to be expected in view of Levene's report that his "cerebronucleoprotein" was characterized by a lower content of phosphorus than other nucleoproteins.

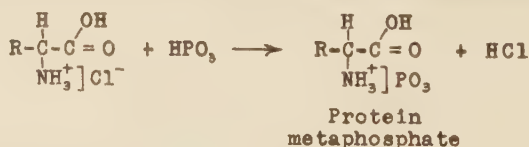
Method C: Preparation of Brain Protein by Precipitation with Metaphosphoric Acid

Metaphosphoric acid, which has long been known to be a good precipitant for proteins, also precipitates the protein-lipoid complex from the aqueous brain sol. A study²⁷ of the action of this reagent on other, pure proteins suggested that its action might be analogous to that of nucleic acid and that the theoretical relationships discovered might be of aid in explaining the nature of the combination of protein with nucleic acid as it exists in the brain protein. The union between metaphosphoric acid and protein is even less ionized than that between nucleic acid and protein. Hence, the use of metaphosphoric acid would have the additional advantage in that the nucleic acid of the nucleoprotein would be replaced, so that this variable factor would be eliminated and the purely protein part of the molecule could be studied. If the lipid-protein combination in acid solution is similar to the nucleoprotein complex, the metaphosphoric acid would also tend to replace the loosely held lipid, thereby making the separation of the lipid from the complex less difficult.

Assuming that the protein molecule has at least one free amino and one free carboxyl group, its reaction with acid may be represented by the equation below, where R is the remaining part of the molecule.



In the study referred to above, it was found that the union between metaphosphoric acid and protein is salt-like, the acid reacting with the basic amino group to form the salt indicated below:



The metaphosphate protein salt is far less dissociated than other

²⁷D. R. Briggs. To be published.

salts formed with acid anions.

The isoelectric points of the metaphosphoric protein compound varied according to the proportions of the constituents present. As the amount of metaphosphoric acid is increased, the isoelectric point shifts toward the acid side. As more basic groups react with addition of more acid, their action becomes masked and the ionization of the compound assumes the character of an acid, the carboxyl groups being unaffected by the metaphosphoric acid. On the alkaline side of the isoelectric point the amino groups do not bind acid and the protein metaphosphate combination breaks down. This was shown in experiments in which the compound was dialyzed at a pH above its isoelectric point. The phosphorus was removed. By electrodialysis or dialysis on the acid side of the isoelectric point only the uncombined phosphorus could be removed. The alkaline dialyzed protein retained its original solubility at its isoelectric point, whereas the protein remained insoluble if the dialysis was carried out at the isoelectric point.

The results of the experiments on the action of metaphosphoric acid on albumin were applied to the isolation of brain protein in the method described below.

A brain sol was prepared as in the first method described. It was treated with a sufficient quantity of freshly prepared 20% solution of metaphosphoric acid to cause the maximum precipitation. The final concentration of acid in the mixture was approximately 2% and the pH was 1.8-2.0. After the mixture had stood overnight at 5°, the clear supernatant liquid was decanted. The precipitate was collected in centrifuge tubes and treated with benzene to reverse the phase. This was more difficult than the reversal of phase of the isoelectric precipitate. In order for a firm paste to be formed, it was necessary to centrifuge the mixture several times so that the excess water could be drawn off. The paste was transferred to bottles and shaken successively with chloroform, ethyl alcohol and ether. This treatment was followed by extraction in a Soxhlet apparatus with chloroform, absolute methyl alcohol and ether. Only a trace of lipid was removed by the extraction with chloroform. No swelling of the protein was observed when methyl alcohol was used as the extractive. The extraction appeared to be complete in a much shorter time than had been required for the extraction of the isoelectric precipitate with this

solvent. The dry, completely extracted preparation contained 6.2% phosphorus.

Three fractions were prepared: I, by extraction with sufficient 0.1 N sodium hydroxide to make a pH of 9.0; II, extraction with 0.1 N sodium hydroxide and III, the insoluble residue. Each fraction was suspended in water and sufficient sodium hydroxide was added to make a pH of about 8.0. The suspensions were placed in collodion membranes and dialyzed against running tap water for three days. Sufficient sodium hydroxide was added to the tap water to maintain it at approximately pH 8.0. During the dialysis it was necessary to add small amounts of alkali to the protein fractions to keep them sufficiently alkaline, since the metaphosphate ions are removed only when the pH of the solution is 2 to 3 pHs higher than the isoelectric point of the protein.

Following the dialysis, each fraction was subjected to electrodialysis to remove all inorganic ions. Electrodialysis was considered to be complete when the current was reduced to a minimum, which required at least 24 hours. During this process the proteins flocculated and the final pH reached was nearly the isoelectric point of the protein.

Each fraction was suspended in a total volume of 50 cc. and titrated electrometrically using a glass electrode. The isoelectric points of each were determined by means of a cataphoresis cell. After the titration, the solutions were made up to 100 cc. and aliquots were used for the determination of the total protein and the phosphorus content. The percentage recovery of each fraction with the phosphorus contents and isoelectric points are given in Table VIII.

TABLE VIII

	Isoelectric Point pH	Phosphorus Content (Percent)	Percentage of Whole Protein in the Fraction
Fraction I	5.10	0.17	1.6
Fraction II	5.35	0.11	33.2
Fraction III	5.35	0.04	32.2

With this method, approximately 35% of the whole protein could be recovered as soluble fractions. A comparison with Table

VI, page 23, shows that this is a somewhat larger recovery of soluble protein than was obtained by Method B, leaving a correspondingly smaller percentage in the insoluble residue. The total recovery was low because of the large number of manipulations involved in the fractionations and dialysis procedures.

The alcohol and ether washings obtained in the drying of the protein fractions prepared by this method were examined for the presence of lipoids. The alcohol washings were evaporated to dryness and the residue from this was treated with hot alcohol and filtered. (This step was necessary since some protein is usually carried over into the first washings.) Only a trace of residue was left from the evaporation of the alcohol. Evidently the removal of lipoids had been more effective in this method than in those previously used.

During the titration of Fraction II it was noted that when about 1.0 cc. of 1.0 N hydrochloric acid had been added, i.e., in the pH range 2.5-3.0, the protein showed a marked tendency to swell and the solution became so viscous that it was difficult to handle. This property had been observed before only when the protein had been treated with 0.1 N alkali.

This suggested that Fraction II was at least partially soluble in acid. A fractionation was carried out to determine whether proteins of different properties could be obtained from it. A portion of the fraction was extracted at pH 3.0. After centrifugation a clear acid solution could be decanted from the portion which was insoluble. The insoluble portion became transparent and gelatinous but did not go into solution when extracted repeatedly at pH 2 to 3. The acid solution was precipitated at its isoelectric point, which was 5.5 (by cataphoresis experiment). The precipitate, designated as II-a, was collected and electro-dialyzed. The portion insoluble in acid, called II-b, was also precipitated and its isoelectric point was found to be pH 4.8. It was electro-dialyzed and both fractions were titrated electrometrically.

The titration curves were constructed by plotting the data from Tables IX to XIII. The fourth column in each of the tables was obtained by calculation, since 1 g. of each fraction was not available. The isoelectric point of each protein was used as the zero abscissa, so that those to the right and left of it represent, respectively, the number of cubic centimeters of 1.0 N alkali and

1.0 N acid required to form a solution of the corresponding pH. For the absolute values of the pH from 9 to 11, corrections should be applied, since the glass electrode gives pH readings which are a little too low in this range. However, the corrections would be approximately the same for each curve and since the curves are of interest only for comparisons of these particular proteins, the corrections were not applied.

The proteins show very little buffer capacity. Fraction II, before the separation into the two components, shows some buffer action in the range, pH 6 to 8, which is not shown by the other fractions.

TABLE IX
ELECTROMETRIC TITRATION OF FRACTION I
Sample used=0.1042 g. of protein in a volume of 50 cc.

Cc. added 0.1 <u>N</u> NaOH	E.M.F. mv.	pH	Cc. 1.0 <u>N</u> NaOH per g. Protein (calcd.)	
0.0	200.2	5.54	0.0	...
5.25 (1 <u>N</u> HCl)	16.3	2.44	...	...
0.0	16.3	2.44	...	...
0.50	19.0	2.49	0.48	4.60
1.00	22.3	2.54	0.96	4.12
1.50	25.7	2.60	1.44	3.64
2.00	29.7	2.67	1.92	3.16
2.50	25.0	2.75	2.40	2.68
3.00	41.6	2.87	2.88	2.20
3.50	49.8	3.00	3.36	1.72
4.00	60.8	3.17	3.84	1.24
4.50	79.4	3.50	4.32	0.76
4.75	94.7	3.74	4.58	0.52
5.00	117.0	4.13	4.80	0.28
5.25	170.1	4.97	5.04	0.04
...	...	(5.10)	(5.08)	Isoelect. point
5.50	276.6	6.80	5.28	0.20
5.75	444.4	9.61	5.52	0.44
6.00	495.0	10.46	5.76	0.68
6.50	527.0	11.00	6.24	1.16

acid
alk.

The isoelectric point was 5.10. This corresponds to 5.08 cc. determined from a graph. This value was used as the zero abscissa of the titration curve.

The E.M.F. reading obtained with a standard buffer solution of pH 4.016 was 130.5 mv. The pH was calculated by the formula:

$$\text{pH} = 4.016 - \frac{130.5}{nRT} + \frac{\text{EMF (mv)}}{nRT}$$

TABLE X
ELECTROMETRIC TITRATION OF FRACTION II
Sample used = 1.0048 g. protein in a volume of 50 cc.

Cc. added 1.0 N NaOH	E.M.F. mv.	pH	
0.0	203.3	5.80	
0.53	73.0	3.40	
0.84	49.5	3.02	
1.37	22.5	2.55	
1.57	14.9	2.42	
1.0 C N HCl			
0.0	14.9	2.42	...
0.20	22.4	2.55	1.33
0.40	32.4	2.72	1.13
0.60	45.6	2.94	0.93
0.80	68.9	3.33	0.73
1.05	91.7	3.73	0.48
1.20	113.3	4.07	0.33
1.30	130.4	4.36	0.23
1.40	152.1	4.73	0.13
1.50	180.0	5.20	0.07
(1.53)	...	(5.35)	Isoelect. point
1.55	199.5	5.53	0.02
1.60	230.3	6.05	0.07
1.65	249.1	6.37	0.12
1.70	276.6	6.82	0.17
1.80	300.0	7.22	0.27
1.90	343.0	7.95	0.37
2.00	425.7	9.34	0.47
2.10	472.0	10.12	0.57
2.20	495.0	10.50	0.67
2.30	510.5	10.76	0.77
2.40	523.0	11.21	0.87

pH 5.35, the isoelectric point, corresponds to the action with 1.53 cc. NaOH which is used as the zero abscissa.

The E.M.F. for the buffer of pH 4.016 was 129.4 mv.

TABLE XI
ELECTROMETRIC TITRATION OF FRACTION III
Sample used = 0.7818 g. protein in a volume of 50 cc.

Cc. added 1.0 <u>N</u> NaOH	E.M.F. mv.	pH	Cc. 1.0 <u>N</u> NaOH per g. protein (calcd.)	
0.0	256.5	6.46	...	
0.53 } 1.0 <u>N</u>	67.5	3.31	...	
1.05 } HCl	33.6	2.75	...	
1.57 }	11.5	2.37	...	
0.0	11.5	2.37	...	...
0.10	12.7	2.40	0.12	1.71
0.30	19.5	2.50	0.38	1.45
0.50	31.0	2.71	0.63	1.20
0.70	44.0	2.91	0.89	0.94
0.90	61.5	3.22	1.15	0.68
1.10	90.0	3.69	1.40	0.43
1.20	111.0	4.04	1.53	0.30
1.30	137.0	4.48	1.66	0.17
1.40	169.4	5.02	1.79	0.04
...	...	(5.35)	(1.83)	Isoelect. point
1.50	221.0	5.89	1.91	0.08
1.60	309.3	7.36	2.04	0.21
1.70	389.3	8.70	2.17	0.34
1.80	454.0	9.80	2.30	0.47
1.90	492.0	10.42	2.43	0.60
2.00	515.0	10.80	2.55	0.72
2.10	529.0	11.04	2.68	0.85

The isoelectric point, pH 5.35, corresponds to 1.83 cc. 1.0 N NaOH per g. of protein. This was taken as the zero point. The E.M.F. of the buffer of pH 4.016 was 130.5 mv.

TABLE XII
ELECTROMETRIC TITRATION OF FRACTION II-a
Sample used = 0.238 g. protein in a volume of 50 cc.

Cc. added 0.1 <u>N</u> NaOH	E.M.F. mv.	pH	Cc. 1.0 <u>N</u> NaOH per g. protein (calcd.)	
0.0	218.4	5.30	...	
5.25 0.1 <u>N</u> HCl	36.8	2.25	...	
0.0	36.8	2.25	...	...
0.50	40.0	2.31	0.21	2.02
1.00	45.2	2.40	0.42	1.81
1.50	49.2	2.47	0.63	1.70
2.00	55.5	2.57	0.84	1.39
2.50	63.4	2.70	1.13	1.09
3.00	73.0	2.86	1.26	0.97
3.50	87.0	3.10	1.47	0.76
4.00	105.5	3.41	1.68	0.55
4.50	133.0	3.87	1.89	0.34
5.00	174.8	4.57	2.10	0.13
5.25	221.5	5.35	2.21	0.02
...	...	(5.50)	(2.23)	Isoelect. point
5.50	273.3	6.23	2.31	0.08
5.75	313.5	6.90	2.41	0.18
6.00	357.5	7.64	2.52	0.29
6.25	446.0	9.12	2.63	0.40
6.50	484.5	9.77	2.73	0.50
6.75	506.6	10.14	2.84	0.61
7.00	516.7	10.31	2.94	0.71
7.50	531.0	10.55	3.15	0.92

acid
↑
↓
alk.

The isoelectric point was 5.50 which corresponds to 2.23 cc. 1.0 N NaOH per g. of protein.

The E.M.F. of the buffer of 4.016 was 141.5 mv.

TABLE XIII
ELECTROMETRIC TITRATION OF FRACTION II-b
Sample used = 0.5344 g. protein in a volume of 50 cc.

Cc. 0.1 N NaOH added	E.M.F. mv.	pH	Cc. 1.0 N NaOH per g. protein (calcd.)	
0.0 5.25 } 0.1 N 7.38 } HCl	207.8 54.5 37.5	5.12 2.55 2.28	...	
0.0	37.5	2.28	...	...
1.0	46.0	2.41	0.18	1.13
2.0	55.6	2.57	0.37	0.94
3.0	68.0	2.78	0.56	0.75
4.0	83.5	3.04	0.75	0.56
4.5	94.1	3.22	0.84	0.47
5.0	105.7	3.41	0.94	0.37
5.5	119.0	3.64	1.03	0.28
6.0	137.0	3.94	1.12	0.19
6.5	159.0	4.31	1.22	0.09
7.0	188.6	4.81	1.31	Isoelect. point
7.5	255.5	5.93	1.40	0.11
8.0	296.0	6.61	1.50	0.19
8.5	352.2	7.55	1.59	0.28
9.0	390.6	8.20	1.68	0.37
9.5	449.0	9.17	1.78	0.47
10.0	484.5	9.77	1.87	0.56
10.5	501.5	10.06	1.97	0.66
11.0	512.5	10.33	2.06	0.75
12.0	535.5	10.64	2.25	0.94

The isoelectric point of the protein was pH 4.79 which corresponds to 1.31 cc. of 1.0 N NaOH per g.

The E.M.F. of the buffer of pH 4.016 was 141.5 mv.

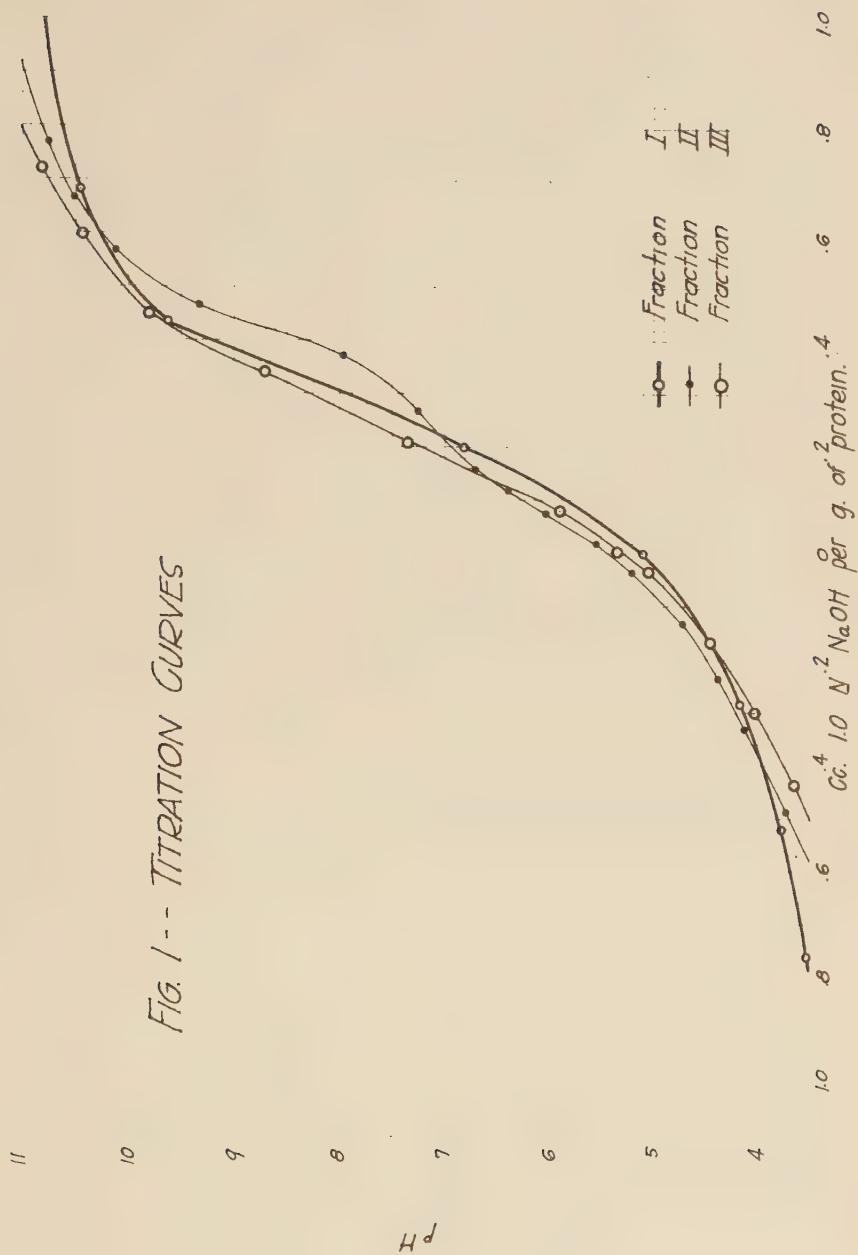
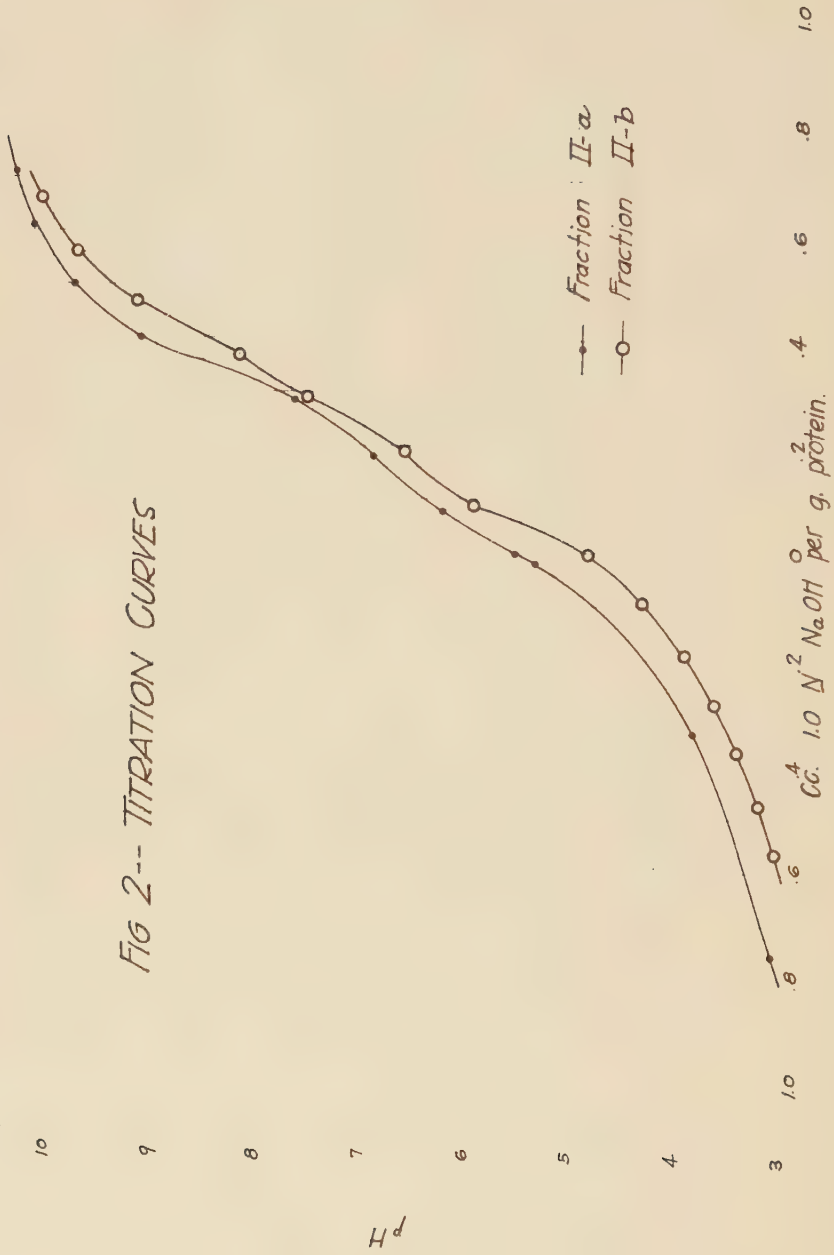


FIG 2-- TITRATION CURVES



PART III
IMMUNOLOGICAL REACTIONS OF THE BRAIN PROTEINS

One of the most sensitive methods for detecting changes in the nature of a whole protein is based upon its ability to act as an antigen. The antigenicity of brain tissue as a whole has been well established. When an emulsion of brain is injected into animals, an antiserum is produced which reacts not only with an emulsion of brain tissue of an animal of the same species, but also with that of brains of other species. The antiserum does not react with emulsions or extracts of other organs²⁸ of any species. It was discovered by Witebsky²⁹ that such an antiserum will react with an emulsion of the alcohol-soluble lipoids of brain as well as with emulsions of whole brain. The lipoids themselves are not antigenic, but if they are activated by the addition of foreign protein, such as serum protein, they are capable of producing antisera which react with an emulsion of whole brain and with the alcohol-soluble lipoids. Since the activation of the alcohol-soluble lipoids can be made only with another protein which is in itself an antigen, it would seem that the brain protein must be an antigen. So far there has been no direct evidence that this is the case.

The only report in the literature of an attempt to determine the antigenic properties of purified brain protein is that of Block and Brand.³⁰ They found that their purified preparations sensitized guinea pigs to anaphylactic shock. Unlike the whole brain, their preparations appeared to be species specific but not organ specific. Since their preparations may have contained small amounts of lipoids, their experiments do not offer conclusive proof that lipid-free brain protein is antigenic.

The method based upon the ability of an antigenic substance to produce an anaphylactic reaction was chosen for the de-

²⁸An exception has been found by J. H. Lewis, J. Immunol., 24, 193 (1933).

²⁹E. Witebsky and J. Steinfeld, Z. Immunitäts., 58, 271 (1928).

³⁰Op. cit.

termination of the antigenic capacity of the brain protein preparations. The Schultz-Dale method³¹ of recording the contractions of non-striated muscles of sensitized animals was used. Virgin guinea pigs were injected intraperitoneally with suspensions of the whole, unfractionated protein and the soluble Fractions I and II, prepared by Method B. The suspensions were prepared by grinding the proteins with 0.05 N sodium hydroxide solution and adjusting the mixture to a final pH of 8.4. The concentration of protein was so adjusted that in each injection the animal received 10 to 15 mg. of the protein. In some cases two or three injections were made at intervals of two or three days. About 10 days after the last injections the animals were killed. The uterine muscle was removed, suspended in a bath containing Locke's solution through which oxygen was bubbled, and attached to the recording device. When the small, normal contractions of the muscle had been recorded the test dose of 3 to 5 mg. of the protein in a solution adjusted to pH 8.4 was added to the solution in the bath. After the reaction to the test dose was recorded, the liquid in the bath was replaced with fresh Locke's solution and a second test dose was added to determine whether the strip had become desensitized. A large, immediate contraction which desensitized the strip so that the second dose caused no response was considered to be a definitely positive reaction.

With the whole brain protein, positive reactions were usually obtained, although some were weak. The results obtained with the soluble Fractions I and II were erratic. In some cases the uterine strips from sensitized animals gave unquestionably positive reactions, in others the response was slight making it difficult to decide whether the reaction was definitely positive and in many cases the reactions were negative. It was concluded that the lipoid-free preparations tested were antigenic but had very low antigenic capacity.

The results of experiments to determine whether Fractions I and II were immunologically identical were also inconsistent. The uterine strips of animals sensitized with Fraction I appeared to react as well to a test dose of protein of Fraction II as to one of Fraction I, but many of the reactions were negative. Similar results were obtained when animals were sensitized with the

³¹H. H. Dale, J. Pharm. Exptl. Therap., 4, 167 (1913).

protein of Fraction II. The experiments gave no evidence of any difference in the immunologic reactions of the two fractions.

Three guinea pigs were also sensitized with the whole unfractionated liver protein (page 28). One strip from each animal was tested with the homologous protein. Positive reactions with complete desensitization were obtained in each case. The liver protein appeared to be a stronger antigen than the brain protein. The second strips from each animal, tested with a solution of the whole brain protein gave no responses and were not desensitized to the action of liver protein. As far as can be judged from this experiment, the brain protein is organic specific.

The antigenicity of the whole protein was also tested by means of the complement fixation reaction. Rabbits were injected subcutaneously with suspensions of the whole, unfractionated protein prepared by Method B. Four injections were made in the course of two weeks. The antiserums obtained gave negative reactions when tested with a solution of the whole protein, solutions of Fractions I and II and with an emulsion of the alcohol-soluble lipoids.

A few complement fixation tests were made with the purified protein which had been prepared by precipitation with metaphosphoric acid. When a solution of this protein was used as the antigen in tests with an antiserum produced by injection of an emulsion of whole brain into rabbits, the reactions were negative.

A possible explanation of the lack of antigenicity may be that the presence of lipoids is essential for the existence of a complete brain antigen. A more probable explanation is that the proteins have become denatured in the processes of isolation and purification.

CONCLUSIONS

The proteins prepared by the first method described, which involved exhaustive extraction of the precipitated protein-lipoid complex, retained a large part of their original solubility. Those prepared by the second method, which involved a reversal of phase and which was more successful than the first in removing the lipoids, had become largely insoluble. The latter preparations showed little, if any, immunizing capacity, giving further evidence that they had become denatured. The proteins prepared by precipitation of the lipoid-protein complex by metaphosphoric acid appeared to be entirely free of lipoids and had not undergone any greater loss of solubility than those prepared by the second method, but were also immunologically inactive. We conclude, therefore, that in the complete dissociation of the lipoid-protein complex, the nature of the protein was so changed that it no longer exhibited the properties which characterize it in its native state.

The experimental studies have shown that the protein of the brain is a mixture of proteins of albuminoid nature with which nucleic acid is conjugated. The protein components differ only slightly from one another in their composition. The proportion of the whole protein, which can be peptized readily, depends upon the method of preparation. Fractions extracted from the whole protein substance at given hydrogen ion concentrations include a mixture of components of very similar composition and properties. That each fraction is, in itself, a mixture is well demonstrated in the fraction extracted with 0.1 N sodium hydroxide from the protein metaphosphate precipitate. By treatment with acid, this fraction can be resolved into two fractions, which have different isoelectric points and whose titration curves are different, although the difference is not marked. Fractions extracted at very different hydrogen ion concentrations show greater differences in composition than those extracted at nearly the same hydrogen ion concentrations. The content of phosphorus does not characterize the protein since it varies with the method used, although there may be a minimum content of phosphorus which is an

elementary part of the molecule.

In view of the complex nature of the protein and of the ease with which it is denatured, the lack of agreement in the results reported in the literature is to be expected. The differences can be attributed to the fact that each investigator was dealing with a mixture, the nature of which depended upon the method he had used in isolating the protein. Levene's claim that only one protein is present was based on the content of phosphorus found and upon his qualitative analyses. Since the difference in the composition of the proteins is probably due to the proportions of the various constituents present rather than to the nature of the chemical groups making up the molecule, his conclusion was justified. McGregor recognized that the brain contained a mixture of proteins and while he concluded that two different soluble proteins could be extracted by neutral and alkaline solvents, he was no doubt dealing with mixtures which were analogous to the Fractions I and II described here. The same comment applies to the proteins prepared by Block and Brand. Their preparations were evidently not denatured to such an extent as the proteins prepared by our methods, since they were capable of immunizing animals. It is very doubtful whether the lipoids could be entirely removed by the method which they describe, hence their preparations may not have undergone such radical changes.

The method described for the reversal of phase of the lipoid-protein complex offers a new method of approach for the separation of the lipoids and proteins. It seems improbable that any denaturation would occur in this change in which the lipoid constituents are made the external or continuous phase. While in this condition, the lipoids can certainly be more easily removed by the appropriate solvents. To remove the lipoids which have a larger number of polar groups, i.e., the cerebrosides, and which therefore are in the same phase as the protein part of the micelle, it becomes necessary to reverse the phase again and to use solvents of a polar nature. It is probable that the changes in the protein occur at this stage. When the nature of the union between the protein and this type of lipoid is more fully understood, means can possibly be found by which the two can be separated without injury to the protein molecule.

Precipitation of the protein with metaphosphoric acid and the subsequent removal of the metaphosphate ion by dialysis is a

method which should find wide application both for the isolation and the purification of proteins.

SUMMARY

Three methods for the preparation of brain protein free from lipoid have been investigated and the properties of the isolated proteins have been described.

A. Proteins were isolated from the isoelectric precipitate of the brain sol by the direct extraction of the precipitate with a series of solvents beginning with polar solvents which would wet the particles of the sol. The final preparations were not entirely free from lipoids. They were partly, although not entirely, denatured. A large fraction of the protein remained soluble.

B. Proteins were isolated from the isoelectric precipitate of the brain sol by extracting the precipitate after the phase had been reversed. The final preparations contained no lipoid, at least not in amounts large enough to be detected. The proteins were largely denatured. A small fraction of the protein which remained soluble gave some indications of a weak immunologic activity.

C. The proteins prepared by the use of metaphosphoric acid as the precipitating agent were also free of lipoid. This method offers an advantage over the Method B in that the lipoids are removed more easily. The proteins were denatured to about the same extent as those described in Method B and gave no immunologic reactions.

I wish to express my sincere thanks to Dr. Julius Stieglitz, who has supervised this investigation and whose kindly interest and advice have been most valuable. I am greatly indebted, and my thanks are herewith tendered, to Dr. H. G. Wells for suggesting the problem and placing at my disposal the facilities for experimentation; to Dr. D. R. Briggs for his kind and unfailing aid in the chemical investigations; and to Dr. J. H. Lewis for his generous assistance in the immunological experiments.

